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β1 Integrin Is Not Essential for Hematopoiesis but Is Necessary for the T Cell-Dependent IgM Antibody Response

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Summary

Several experimental evidences suggested that β1 integrin-mediated adhesion of hematopoietic stem cells (HSC) is important for their function in the bone marrow (BM). Using induced deletion of the β1 integrin gene restricted to the hematopoietic system, we show that β1 integrin is not essential for HSC retention in the BM, hematopoiesis, and trafficking of lymphocytes. However, immunization with a T cell-dependent antigen resulted in virtually no IgM production and an increased secretion of IgG in mutant mice, while the response to a T cell-independent type 2 antigen showed decreases in both IgM and IgG. These data suggest that β1 integrins are necessary for the primary IgM antibody response.

Introduction

All blood cells derive from hematopoietic stem cells (HSC), which have the potential to self-renew and to differentiate into all hematopoietic lineages. Cell-cell and cell-matrix adhesion of hematopoietic cells are important to control their migration, retention, self-renewal, and differentiation (Prosper and Verfaille, 2001). Integrins are an important family of cell surface receptors mediating several of these interactions. Integrins are transmembrane molecules consisting of an α and a β subunit. They bind extracellular matrix (ECM) components such as fibronectin and laminin, but also cellular receptors such as VCAM-1 (Hynes, 1992). The cytoplasmic domain is connected to the actin cytoskeleton. Ligand binding to integrin activates various intracellular signaling pathways, which results in cytoskeletal reorganization and changes in gene expression affecting proliferation, differentiation, and survival of cells (Giancotti and Ruoslahti, 1999). Intracellular events, on the other hand, can modulate the affinity/avidity of integrins, which is crucial for the extravasation of leukocytes (Gonzalez-Amaro and Sanchez-Madrid, 1999).

HSC express several integrins that play a prominent role in their adhesion to ECM and nonhematopoietic cells of the bone marrow (BM) and perhaps also in their proliferation. In vitro experiments demonstrated that adhesion of HSC and hematopoietic progenitor cells (HPC) to fibronectin is mediated by α4β1 as well as α5β1 (Williams et al., 1991; van der Loo et al., 1998). α4β1 also mediates binding to VCAM-1 expressed on BM stroma cells (Oostendorp et al., 1995). The importance of these interactions was confirmed by intravenous (i.v.) injection of FN fragments and antibodies against α4β1 or VCAM-1, which mobilized HPC into the blood (Papayannopoulou, 1995; van der Loo et al., 1998). In addition to retention of HSC and HPC in the BM, integrin-mediated adhesion might also be crucial for the self-renewal and survival of HSC, since α4β1-mediated attachment of HPC to fibronectin promotes proliferation (Yokota et al., 1998; Schofield, 1998) and prevents apoptosis (Wang et al., 1998).

Genetic studies with α4 null chimeric mice revealed an important function of α4 integrin in myelo- and lymphopoiesis. Since the α4 subunit can associate with either the β1 or the β7 subunit, α4 null hematopoietic cells lack both α4β1 and α4β7. They have severe defects in the development of erythroid, myeloid, and B cell progenitors (Arroyo et al., 1996, 1999). In addition, T cell precursors are unable to leave the BM. None of these defects was observed in β7 null mice, which lack α4β7 but still express α4β1 (Wagner et al., 1996). It was therefore concluded that α4β1 and not α4β7 is responsible for the marked abnormalities in α4 null chimeric mice. A plausible explanation for the severe phenotype is a defect of α4 null progenitors in the transmigration through the BM stroma and in proliferation (Arroyo et al., 1999).

Integrin expression furthermore affects the migration and function of differentiated blood cells as highlighted by the severe defect in granulocyte extravasation in patients lacking β2 integrin (Hogg and Bates, 2000). It is not known whether β1 integrin plays a role for the normal circulation of lymphocytes, although it is crucial for the extravasation of β1 null HSC into hematopoietic organs (Hirsch et al., 1996; Potocnik et al., 2000). With respect to lymphocyte function, both costimulatory (Yamada et al. 1991; Damle and Aruffo, 1991) and inhibitory effects (Groux et al., 1989; Ticchioni et al., 1993) of β1 integrin on T cell activation were demonstrated in vitro. The exact function in vivo, however, is unclear.

We generated mice in which we induced a deletion of the β1 integrin gene restricted to the hematopoietic system to directly study the role of β1 integrin for HSC function as well as for migration and function of differentiated blood cells.

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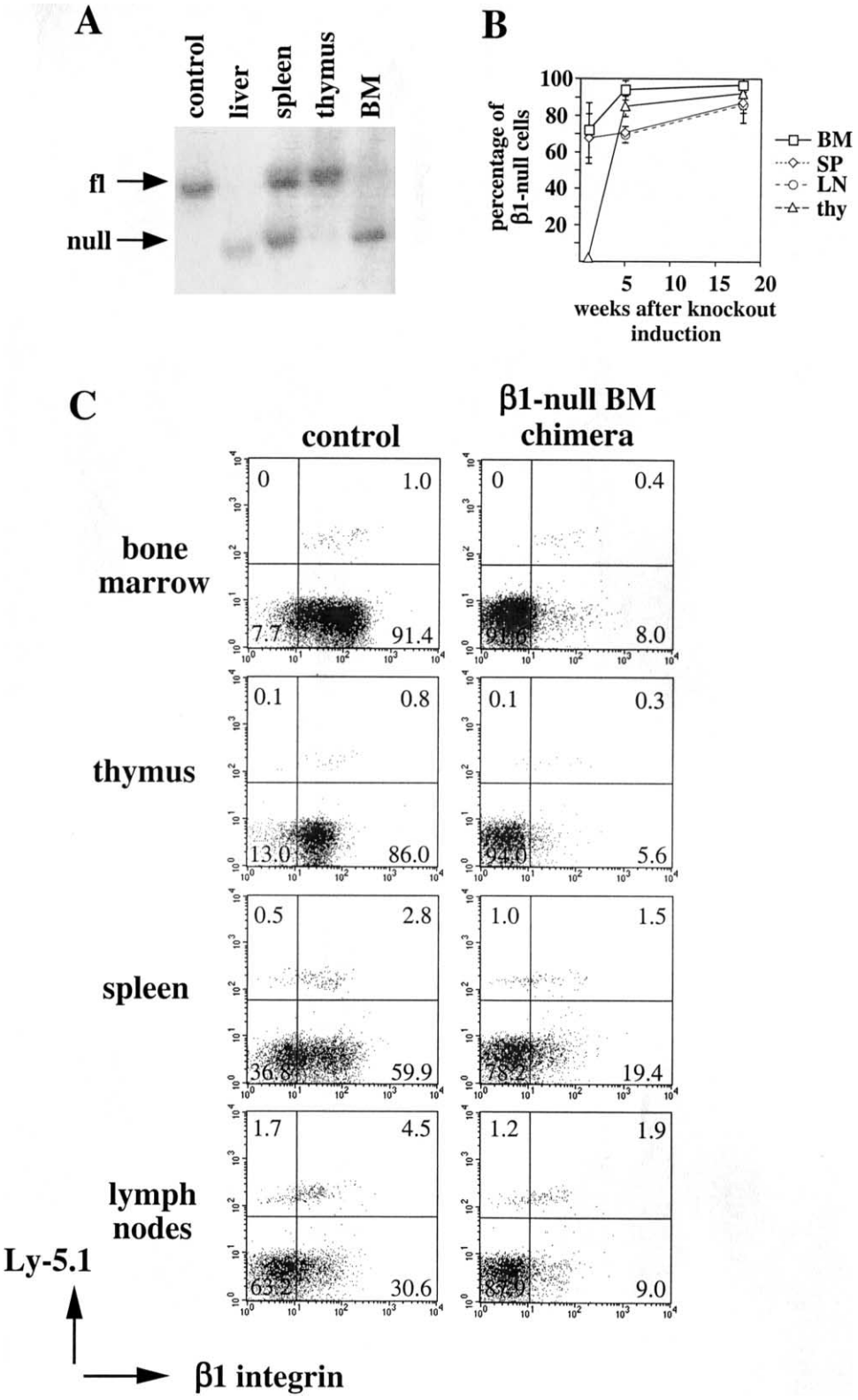

Figure 1. Efficient Deletion of the β1 Integrin Gene in the Hematopoietic System

(A and B) Southern blot analysis of genomic DNA isolated from indicated organs of (fl/fl cre) mice or β1 null BM chimera 1 week (A) or at different time points (B) after the first polyIC induction. Liver DNA from (fl/+) mice was used as a control. A lacZ fragment was used to identify the floxed and the null allele.

(C) Cells were isolated from various lymphoid organs of (fl/– cre) and control (fl/+ cre) BM chimeras 4 months after induction of the knockout. Cells were analyzed by FACS for expression of β1 integrin and of Ly-5.1, which specifically recognizes cells derived from the host (n = 3-6/3-6).

Results

Maintenance and Differentiation of β1 Null HSC

β1 integrin function is essential for homing of adult HSC to the BM (Potocnik et al., 2000). To test whether β1 integrin is important for the function of HSC within the BM, we induced the deletion of the β1 integrin gene in the murine hematopoietic system.

Mice carrying a β1 integrin gene flanked by loxP sites (fl/fl) (Potocnik et al., 2000) were mated with mice with a neomycin-disrupted β1 gene (+/–) (Fässler and Meyer, 1995) and mice expressing the cre recombinase under the control of the Mx promoter (+/+ cre) (Kühn et al., 1995) to obtain a mouse strain with a conditional and a null allele for β1 integrin as well as the cre transgene (fl/– cre). These mice expressed β1 integrin on hematopoietic cells and were phenotypically normal. Three injections of polyIC at 2 day intervals 4–8 weeks after the BM transfer resulted in an efficient deletion of the β1 integrin gene in liver and BM within 1 week (Figures 1A and 1B). The deletion in spleen was about 70%, while less than 5% of the thymocytes had lost the β1 integrin gene.

To restrict the deletion of the β1 integrin gene to the hematopoietic system, BM from noninduced (fl/– cre) mice was transferred into lethally irradiated B6SJL mice. As control, BM from litter mates carrying a conditional and a wild-type allele of β1 integrin as well as the cre transgene was transferred to recipient mice. After reconstitution of the hematopoietic system, the mice were treated with polyIC. Mice were analyzed by FACS 2, 4, 6, and 8 weeks and 3, 4, 6, 8, 10, and 12 months after the polyIC treatment.

After 8 weeks, BM cells and thymocytes had lost nearly the entire β1 integrin expression, while spleen, lymph nodes, and Peyer's patches contained about 15% β1-expressing cells (Figures 1B and 1C).

The percentage of host-derived cells was determined by host (Ly- 5.1)- and donor-specific (Ly-9.1, Ly-5.2) markers. Four months after knockout induction, the host cell contribution was 1% or less in thymus and bone marrow and up to about 5% in spleen, lymph nodes, and Peyer's patches (Figure 1C and data not shown). These host cells were almost exclusively T cells leading to a host cell contribution to the $CD3^+$ T cells of about 15% in BM and 10% in spleen and lymph nodes. In all these tissues, the host cell contribution to B cells was lower than 1%. More than 95% of the BM cells expressing markers for the erythroid (Ter-119) and myeloid (Gr-1, Mac-1) lineage lacked expression of β1 integrin. The population sizes were similar to normal mice (data not shown). Differential blood counts revealed similar numbers of polymorphonuclear (PMN) and mononuclear cells (M) in mutant and control mice 5 weeks and 4 months after induction of the knockout (Table 1). This phenotype was also observed even 12 months after the induction of the knockout, further corroborating that the β1 integrin gene was deleted on HSC and that loss of β1 integrin expression did not impair HSC function in the BM.

To assess the number of granulocyte/macrophage and erythroid progenitor cells in the BM colony, assays (GM) were carried out with BM leukocytes. 5 weeks and 4 months after induction of the knockout, the frequency of GM HPC in BM was similar in mutant and control mice (Table 2). FACS analysis of randomly picked colonies indicated an efficient deletion of the β1 integrin gene on GM HPC. 36 out of 37 colonies derived from 4 different mutant mice 5 weeks after knockout induction were β1 deficient. After 4 months, all 49 colonies derived from 5 different mutant mice lacked expression of β1 integrin. To test whether β1 null BM chimera show an increased release of HPC into the blood, we performed colony assays (GM) with peripheral blood leukocytes isolated from mice 2 weeks after induction of the knockout. At this time, more than 98% of the platelets in the mutant mice had lost β1 integrin expression (data not shown). The frequency of colony forming units in the peripheral blood was similar in normal and mutant BM chimera (Table 2), indicating an efficient retention of progenitor cells in the BM of mutant mice.

Table 1. Normal Numbers of Polymorphonuclear and Mononuclear Cells in the Peripheral Blood

	Control		β1 Null BM Chimera		
	PMN	M	PMN	M	n/n
5 weeks	1.48 ± 0.61	2.15 ± 0.57	1.83 ± 1.14	2.4 ± 0.74	4/4
4 months	0.89 ± 0.29	1.39 ± 0.75	0.97 ± 0.61	1.77 ± 0.71	6/6

5 weeks and 4 months after induction of the β1 integrin gene deletion, whole blood of control and β1 null BM chimera was diluted 1:10 with Türk stain and differentially counted in a hematocytometer. n/n indicates the number of animals in control and mutant group. (All numbers in 10^6 cells/ml). PMN, polymorphonuclear; M, mononuclear.

B Cell Development in the Absence of β1 Integrin

α4 integrin-deficient mice, which lack α4β1 and α4β7, have a severe defect in B cell differentiation (Arroyo et al., 1996, 1999). Since β7 null mice have normal B cell development (Wagner et al., 1996), α4β1 integrin was thought to cause the B cell defect. Therefore, we monitored B cell development in BM, spleen, and lymph nodes of β1 null BM chimera using different B cell-specific markers: B220 (pre-proB and later), CD19 (proB and later), CD25 (preBII), IgM (immature B), CD21, CD23, and IgD (all mature B).

Table 2. Normal Number of Progenitor Cells in Blood and BM of β1 Null BM Chimera

			Control	β1 Null BM Chimera	n/n
2 weeks	PB	GM	5.5 ± 4.0	3.8 ± 5.0	5/7
5 weeks	BM	GM	37.7 ± 6.8	47.1 ± 11.7	4/4
		preB	20.6 ± 9.5	22.3 ± 13.5	4/4
4 months	BM	GM	49.2 ± 18.3	43.7 ± 2.8	5/5
		preB	10.5 ± 1.6	13.1 ± 8.7	5/5

Methylcellulose colony assays for granulocyte, macrophage, and erythrocyte progenitors (GM) were carried out with 200000 leukocytes from peripheral blood (PB) 2 weeks after knockout induction or with 20000 cells of BM 5 weeks and 4 months after knockout induction. For preB cell assays, 50000 BM leukocytes were seeded per dish. The assay was carried out in triplicates and colonies were counted after 8–9 days. Presented is the number of colonies per dish with standard deviation. n/n indicates the number of animals in control and mutant group. Integrin expression was assessed by FACS on randomly picked clones (see Results).

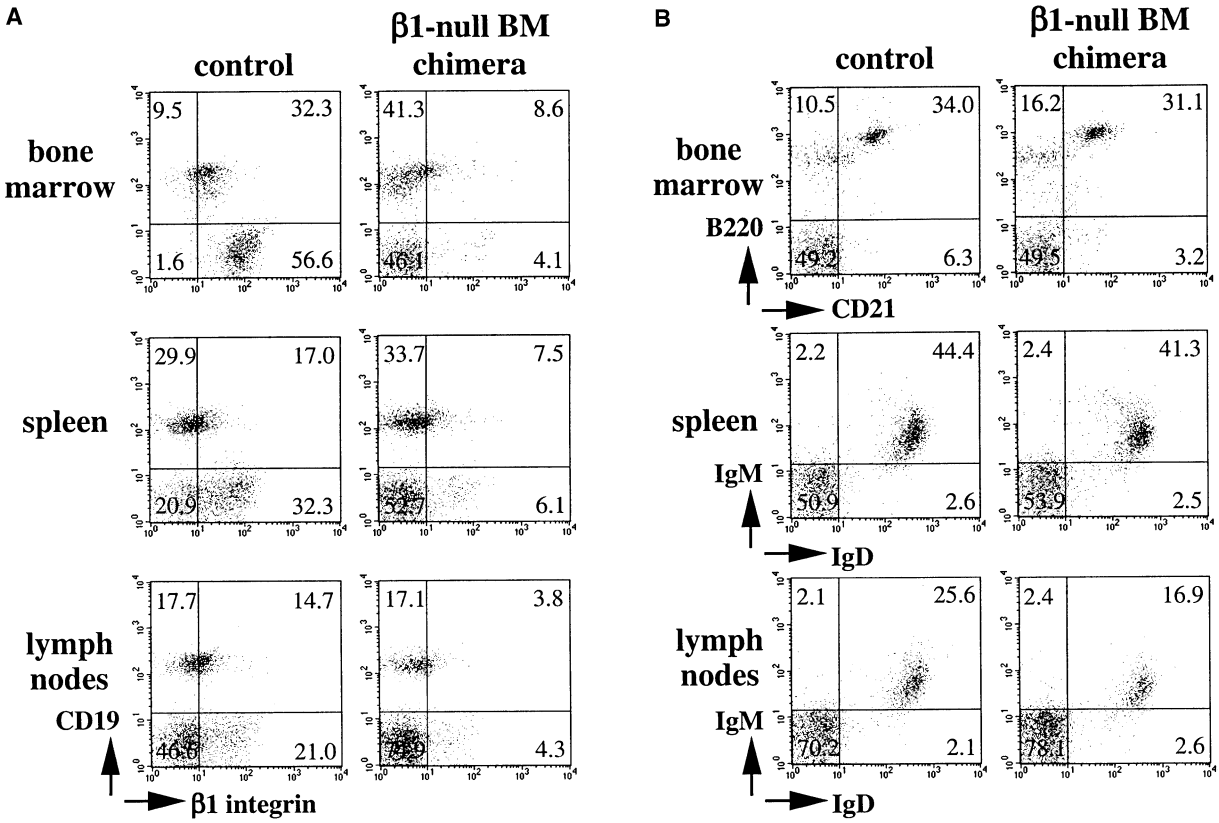

Figure 2. Normal B Cell Development in the Absence of β1 Integrin

(A) Presence of β1 null $CD19^+$ B cells in BM, spleen, and lymph nodes of β1 null BM chimeras 4 months after induction of the knockout (n = 5-6/4-6).

(B) Normal sizes of B cell subsets characterized by the expression of the B cell markers B220, CD21, IgM, and IgD in BM, spleen, and lymph nodes of β1 null BM chimeras 4 months after induction of the knockout as determined by FACS (n = 5-6/4-6).

The frequency of preB cells in BM was determined in methylcellulose assays. Similar numbers of preB cells were found in BM of mutant and control mice 5 weeks and 4 months after induction of the knockout. FACS analysis of randomly picked colonies indicated a loss of β1 integrin expression on about 95% of the preB cells. Out of 20 colonies analyzed from 4 different mutant mice 5 weeks after induction of the knockout, 19 lacked expression of β1 integrin (Table 2). After 4 months, 18 out of 19 preB cell colonies derived from 5 different mutant mice were β1 deficient.

85% or more of the $CD19^+$ cells in the mutant BM, spleen, lymph nodes, and Peyer's patches showed no detectable expression of β1 integrin after 5 weeks and at all later time points tested (Figure 2A and data not shown). In most cases, similar numbers of $B220^{medium}$ (immature), $B220^{high}$ (mature), $CD25^+$, and IgM^+ cells were found in BM of control and mutant mice, although the relative amounts of immature and mature cells varied between different BM transplantations, both in control and mutant mice (Figure 2B and data not shown). Mutant and control mice had similar amounts of IgD^+IgM^+ cells in spleen and lymph nodes (Figure 2B). In spleen, the levels of $B220^+CD21^+CD23^+$ and $B220^+CD21^+CD23^-$ cells (the latter characterizing marginal zone B cells) were similar in mutant and control mice 5 weeks and 4 months after knockout induction (data not shown).

Next we tested whether the loss of β1 integrin resulted in a compensatory upregulation of other integrin subunits on immature ($B220^{medium}$) and mature ($B220^{high}$) B cells in the BM. Normal immature B cells expressed α4, α5, low amounts of α6, β1, β2, but no α1, α2, β3, and β7 (data not shown). Normal mature B cells expressed α4, low amounts of α6, reduced β1, low and high amounts of β2, and β7 integrin, but not α1, α2, α5, and β3. Deletion of the β1 gene on immature B cells resulted in loss of α4 and α5 integrin expression, whereas on mature B cells α4 expression was only slightly reduced (data not shown).

Transient Defect in Thymus Colonization but Normal Intrathymic Differentiation

Previous reports suggested that α4 integrin is essential for the emigration of T cell precursors from the BM (Arroyo et al., 1996), α6β1 integrin participates in their migration to the thymus (Ruiz et al., 1996), and α4β1 and α5β1 are important for intrathymic development (Salomon et al., 1994; Crisa et al., 1996). Therefore, we assessed T cell development in β1 mutant mice.

One week after induction of the knockout, less than 5% of the thymocytes had lost the β1 integrin gene compared to more than 75% of the BM cells (Figures 1A and 1B). In the following weeks, the percentage of β1 null thymocytes slowly increased to about 95% after

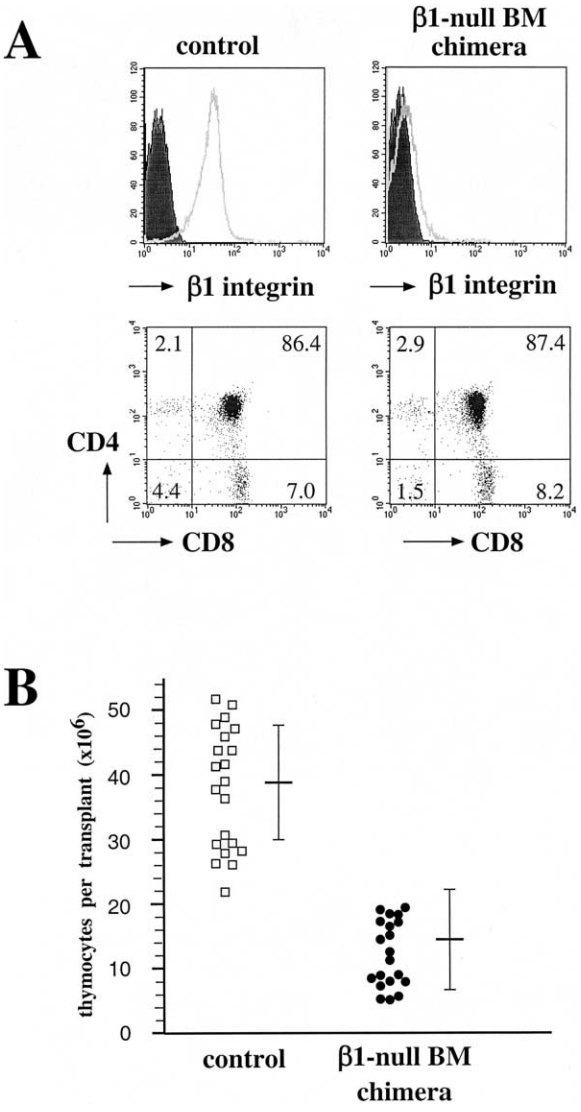

Figure 3. T Cell Precursors Can Migrate to the Thymus and Develop into T Cells in the Absence of β1 Integrin

(A) Efficient deletion of β1 integrin expression on thymocytes and normal development to CD4CD8 DP, CD4 SP, CD8 SP cells in the thymus of β1 null BM chimeras 4 months after induction of the knockout (n = 3/3).

(B) T cell depleted fetal thymi from normal mice were engrafted under the kidney capsule of normal and β1-deficient BM chimera. 4–5 weeks after engraftment of the thymi into β1 null or control BM chimeras the mice were sacrificed and the cell numbers within the transplanted thymi were counted. β1 null T cell precursors could migrate but less efficiently into thymi engrafted under the kidney capsule.

about 6 weeks as determined by FACS analysis. The subset sizes of CD4CD8 DN, CD4 SP, CD8 SP, and CD4CD8 DP cells were similar in mutant and control mice at all time points tested (Figure 3A and data not shown). Interestingly, mutant mice displayed a transient reduction of the number of thymocytes. 5 weeks after induction of the gene ablation, mutant thymi contained $56.8 \pm 6.0 \times 10^6$ cells, whereas thymi from control mice contained $87.1 \pm 6.0 \times 10^6$ cells, indicating a 35% reduction of thymic cellularity in β1 null BM chimera. After 8 weeks, this difference disappeared and the cell numbers were similar in control and knockout mice.

To confirm and further investigate the migration of T cell precursors to the thymus and the transient reduction in thymocytes, we performed an additional experiment. Fetal thymi were isolated, depleted of T cells, and engrafted under the kidney capsule of β1-deficient BM chimera. After 4 weeks, thymocytes were isolated and counted. β1 null thymocytes were present in the engrafted thymi, confirming that β1 null T cell precursors from the BM can colonize the thymic tissue (Figure 3B). In agreement with the transient reduction of thymocytes observed in the endogenous thymus, the number of cells per ectopic thymus was about 3-fold lower in β1 null BM chimera. Thymocytes were then analyzed by FACS using different markers for the intrathymic T cell development (CD25, CD44, CD4, CD8, TCR). CD4CD8 DN cells were subdivided into ($CD25^-$ $CD44^+$), ($CD25^+$ $CD44^+$), ($CD25^+$ $CD44^{lo}$) and ($CD25^-$ $CD44^-$) subsets. In addition to CD4 CD8 DP, CD4 SP, and CD8 SP, also the TCR^{dull} and TCR^{high} population sizes were analyzed. No difference was found in the relative population size of these thymocyte subsets between normal and mutant mice (data not shown), indicating normal intrathymic development of T cells.

Activated Lymphocytes

β1 integrins have a costimulatory role on T lymphocytes in vitro (Yamada et al. 1991; Damle and Aruffo, 1991). Therefore, we examined whether loss of β1 integrin affects the amount of activated lymphocytes in nonimmunized mice. We analyzed the expression of the activation markers CD62L, CD44, and CD69 on B cells and $CD4^+$ and $CD8^+$ T cells in spleen, lymph nodes, and peripheral blood. Activated lymphocytes express low levels of CD62L and high levels of CD44 and CD69. No significant difference in the amount of activated lymphocyte subsets was observed between mutant and normal mice (data not shown).

Homing of Lymphocytes to Spleen, Lymph Nodes, and Peyer's Patches

Since β1 integrin is crucial for the extravasation of hematopoietic stem cells, we tested whether it also plays a role in the migration of differentiated lymphocytes to spleen, lymph nodes, and Peyer's patches. In addition, we analyzed the distribution of the β1-deficient B and T cells within these organs.

In competitive homing assays, mixtures of mutant and wild-type lymphocytes were injected into the tail vein of recipient mice. After 4 and 16 hr, the relative amounts of mutant and normal transferred cells in the different organs were determined by FACS. B and T cells of control, knockout, and recipient mice were distinguished by cell surface markers and by labeling cells with CMTMR prior to injection.

β1 null B cells showed normal homing to spleen, lymph nodes, and Peyer's patches 4 hr as well as 16 hr after injection (Figure 4A). Similarly, T cell migration to secondary lymphoid organs was not significantly affected by the loss of β1 integrin (Figure 4B). Labeling by CMTMR did not affect the homing efficiency. Homing

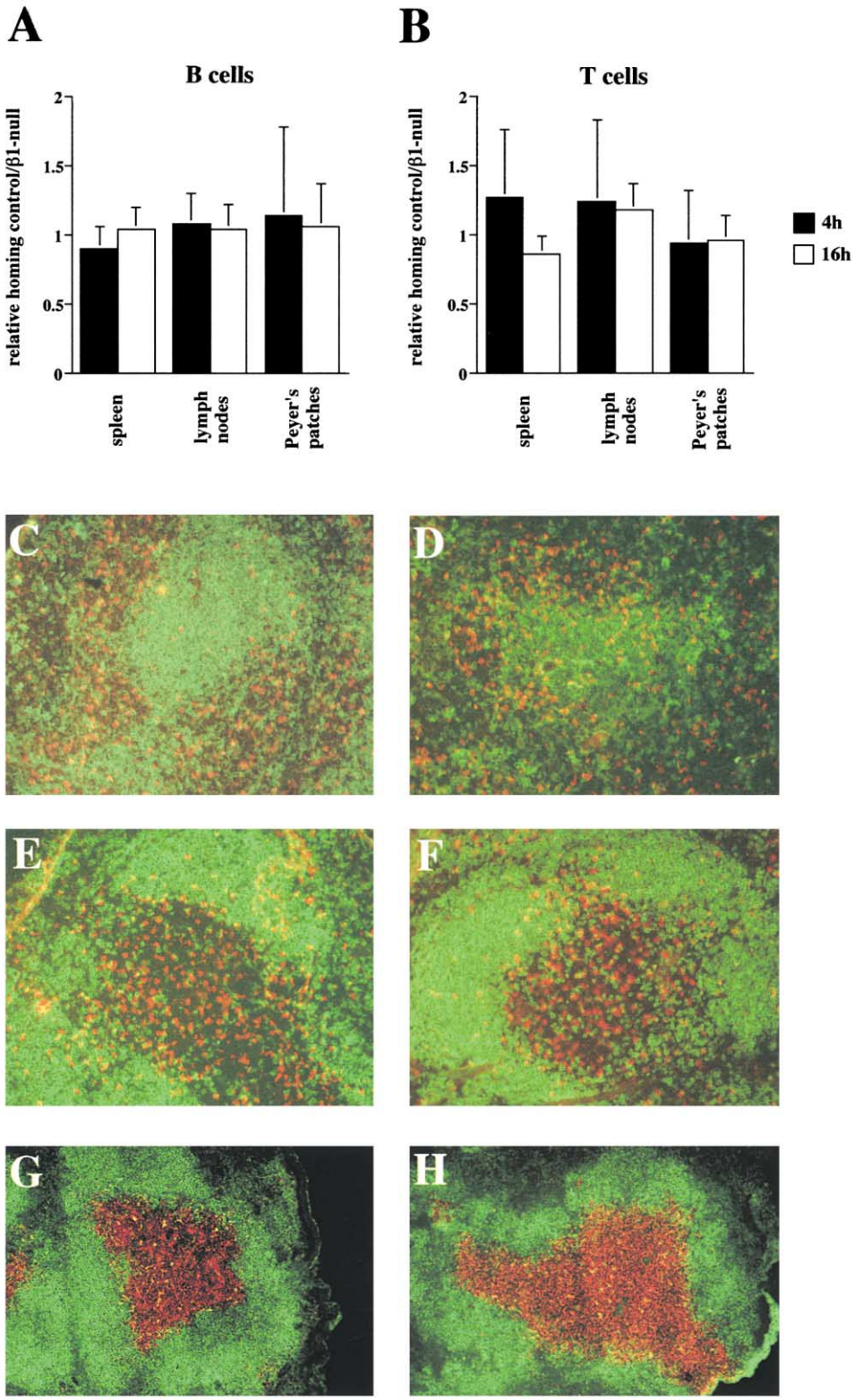

Figure 4. Normal Homing of β1 Null B and T Cells into Spleen, Lymph Nodes, and Peyer's Patches

(A and B) Mixtures of equal amounts of control and β1 null cells (both Ly 5.2), one of them labeled with the intracellular fluorescent dye CMTMR, were injected into the tail vein of B6SJL recipient mice (Ly-5.1). After 4 and 16 hr the mice were sacrificed. The ratio of control to β1-deficient cells in spleen, lymph nodes, and Peyer's patches was determined by FACS (n = 3-5). These numbers were divided by the ratio of control to mutant cells in the injected cell mixture, as assessed by FACS, resulting in the relative homing of control to mutant cells. Error bars indicate standard deviation.

assays with enriched preparations of B and T cells showed similar results.

Exchange of dyes or separation of B and T cells using B220 or Thy-1.2 coupled magnetic beads showed similar results.

To study the localization of transferred lymphocytes within the spleen, B and T cell fractions of splenocytes from control and mutant mice were injected into the tail vein of B6SJL mice. Spleen sections were stained with Ly-5.2, detecting specifically the transferred cells. To reveal the tissue organization, sections with labeled B and T cells were counterstained with Thy-1.2 or B220, respectively. After 4 and 16 hr, β1 null and normal B cells were mainly found outside the CD3-rich periarteriolar lymphoid sheath (PALS) (Figures 4C and 4D; data not shown). At the same time points, mutant and wild-type T cells were found concentrated in the PALS region (Figures 4E and 4F; data not shown).

To detect long-term defects or defects dependent on the interactions of lymphocytes with other β1-deficient hematopoietic cells, we assessed the distribution of B and T cells in spleen, lymph nodes, and Peyer's patches of normal and β1 null BM chimera. Mutant mice showed normal distribution of B and T cells within the lymphoid organs (Figures 4G and 4H; data not shown).

Decreased Amount of IgM but Increased Levels of IgG after T Cell-Dependent Immunization of Mutant Mice

To study whether the loss of β1 integrin in the hematopoietic system affects the antigenic response, normal and mutant mice were immunized with the T cell-dependent (TD) antigen DNP-OVA. The number of germinal centers per splenic section and the accumulation of T cells in B follicles was similar between mutant and wild-type mice 7 days after immunization (data not shown). However, the relative titer of IgM was dramatically reduced in the β1 null mice 7, 14, and 21 days after immunization (Figure 5A). IgG levels were slightly higher in the β1 null BM chimera, indicating that lack of β1 integrin was not associated with a general attenuation of the immune response (Figure 5B). A more detailed analysis showed that both IgG1 and IgG2a were similarly increased in the sera of mutant mice, while IgG3 was decreased (Figure 5C). Immunofluorescence analysis of spleen sections revealed a significant reduction in IgM plasma cells in spleen of β1 null BM chimera, but no obvious difference in their localization (Figures 5D and 5E). Antigen specificity of the plasma cells was assessed in NP-Ova immunized mice. We found small numbers of NP-specific plasma cells in the β1 null BM chimera that were scattered in the red pulp and did not form foci associated with splenic vasculature as seen in normal mice (data not shown).

Decreased Levels of Both IgM and IgG after Immunization of Mutant Mice with a T Cell-Independent Type 2 Antigen

Immunization with the type 2 T cell-independent (TI-2) antigen DNP-Ficoll led to decreased levels of both IgM and IgG in the β1 null BM chimera 7, 14, and 21 days after treatment (Figures 6A and 6B). IgG subtype analysis revealed that IgG3, the major isotype produced in response to T cell-independent type 2 antigens, but also IgG1 and IgG2a, are decreased in the mutant mice (Figure 6C).

Normal Secretion of Both IgM and IgG by Mutant B Cells after LPS Treatment In Vitro

To test whether the reduced production of IgM in mutant mice is a B cell autonomous defect, splenic leukocytes were stimulated in vitro with 10 μg/ml lipopolysaccharide (LPS), which directly activates B cells. After 3 and 6 days, normal levels of IgM and IgG were detected in the supernatant of mutant splenocytes, indicating that the impaired IgM response observed in vivo is caused by defective B cell activation and not by an intrinsic B cell defect that prevents the efficient production of IgM (Figure 6D).

Discussion

Retention and Self-Renewal of HSC

It has been suggested that β1 integrins on HSC are important for HSC function (Prosper and Verfaille, 2001). We tested this hypothesis by inducing a deletion of the β1 integrin gene on HSC in the BM of adult mice. Southern blot and FACS analysis revealed a swift and efficient loss of the β1 gene and protein, respectively. The mutant mice, however, showed normal hematopoiesis, normal number of BM cells, normal retention of HSC in the BM, and an unchanged percentage of β1-deficient BM cells even 12 months after induction of the knockout.

In contrast to our findings, antibody and peptide inhibition experiments had previously suggested that α4 integrin, and to a lesser degree also α5 integrin (Papayannopoulou, 1995; van der Loo et al., 1998) and CD44 (Verfaillie et al., 1994; Vermeulen et al., 1998), are involved in the retention of HPC and HSC in the BM. However, such blocking studies bear the risk of unwanted side effects, e.g., by steric hindrance of other ligand interactions or by partial activation of the target cell. Furthermore, antibodies and protein fragments injected into the tail vein of mice can bind to many other integrin-expressing cells before reaching the HSC in the BM. It could be, therefore, that such interactions indirectly favored the release of HSC from the BM.

Studies of α4-, α5-, or αv null chimeric mice, respectively, revealed that none of these integrins alone is

(C–F) B cells (C and D) and T cells (E and F) from control (C and E) and mutant mice (D and F; all Ly-5.2) were injected into the tail vein of B6SJL recipient mice (Ly-5.1). 4 hr later, the mice were sacrificed and spleen sections stained with antibodies against Ly-5.2 detecting the transferred cells (red) and counterstained with Thy-1.2 (green; C and D) or antibodies against B220 (green; E and F). β1-deficient cells showed a similar distribution as transferred normal cells (200× magn.).

(G and H) Spleen sections of normal (G) and β1 null BM chimeric mice (H; 6 months after induction of the knockout) were stained with antibodies against B220 (green) and Thy-1.2 (red), detecting B and T cells, respectively. B and T cells show a normal distribution (100× magn.).

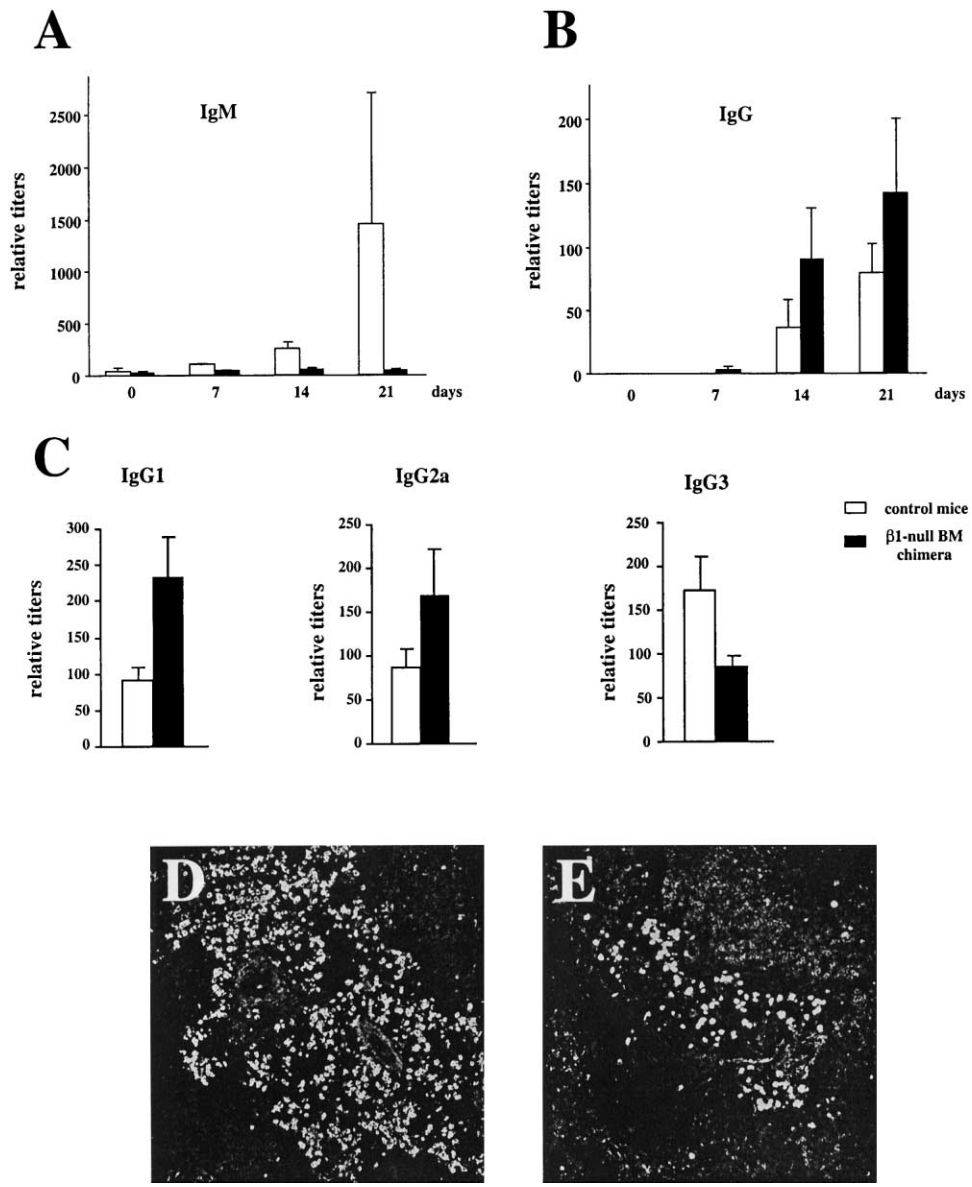

Figure 5. Decreased IgM and IgG3, but Increased IgG, IgG1, and IgG2a Production after Immunization of Control and β1 Null BM Chimera with a T Cell-Dependent Antigen

(A–C) Control (n = 5) and β1 null BM chimera (n = 5) were i.p. injected with DNP-OVA. Serum levels of DNP-specific IgM (A) and IgG (B) were determined by ELISA after 7, 14, and 21 days. Serum levels of IgG1, IgG2a, and IgG3 were determined after 21 days (C).

(D and E) Spleen sections of normal (D) and β1 null BM chimeric mice (H) 7 days after immunization with DNP-OVA stained for IgM (200× magn.).

essential for the retention of HSC in the BM (Arroyo et al., 2000). We extend these studies and show that even simultaneous loss of both fibronectin receptors α4β1 and α5β1 in β1-deficient HSC did not impair HSC retention and function. Although β1 integrin is apparently not necessary for HSC retention, it cannot be excluded that the retention is reduced in the β1 null BM chimera leading to an increased amount of HSC leaving the BM. However, since loss of β1 integrin on HPC did not lead to a decreased number of colony forming units (CFU) in BM or an increased number of CFU in PB, this possibility does not seem likely. These data suggest that mutant HSC use additional adhesive mechanisms mediated for example by the fibronectin and hyaluronan receptor CD44, which itself is not crucial for HSC retention and maintenance as revealed in mice lacking the CD44 gene (Schmits et al., 1997). It is possible that these alternative adhesive mechanisms are not important under normal conditions but are used in the mutant mice to compensate for the loss of β1 integrins. Double mutants should allow testing of whether such compensatory mechanisms are activated in our mouse strain.

Several earlier reports suggested that hematopoietic progenitor cell attachment to fibronectin mediated by α4β1 integrin affects their proliferation and survival in vitro, although differently in different assay systems (Schofield et al., 1998; Yokota et al., 1998; Hurley et al., 1995; Wang et al., 1998). It was speculated that these effects also play an important role in the self-renewal and proliferation of HSC. However, we did not observe any

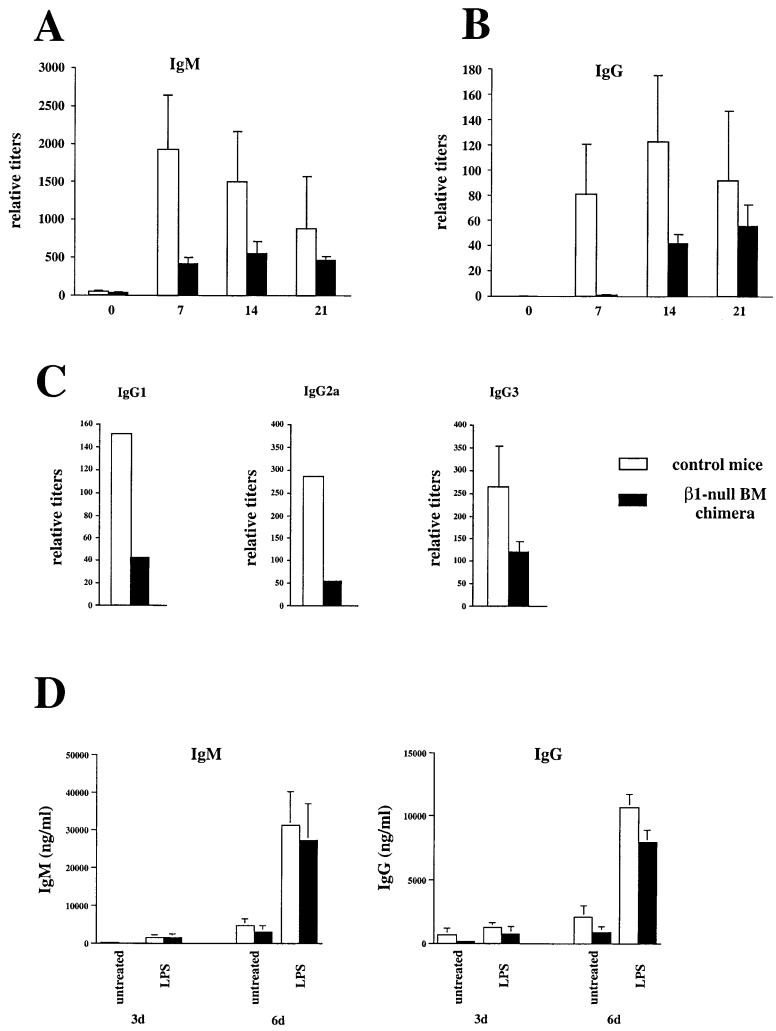

Figure 6. Decreased IgM, IgG, IgG1, and IgG3 Production after Immunization of Control and β1 Null BM Chimera with a T Cell-Independent Type 2 Antigen

(A–C) Control (n = 4) and β1 null BM chimera (n = 5) were i.p. injected with DNP-Ficoll. Serum levels of DNP-specific IgM (A) and IgG (B) were determined by ELISA after 7, 14, and 21 days. Serum levels of IgG1, IgG2a and IgG3 were determined after 21 days (C).

(D) Splenic leukocytes from control (n = 6) and mutant animals (n = 6) were incubated for 3 and 6d in the presence or absence of 10 μg/ml LPS. Cell supernatants were tested for IgM and IgG levels by ELISA. Error bars indicate standard deviation.

changes in the β1-deficient mice in vivo, where the presence of β1 null HSC and progenitor cells was indicated by the generation of mutant B and T lymphocytes, granulocytes, erythrocytes, and platelets even 12 months after deletion of the β1 gene. Although we could not detect any change in the size of B cell subsets in the BM, homeostatic mechanisms might conceal different proliferation rates in the mutant animals (Agenes et al., 2000).

Lymphopoiesis

The analysis of α4 null chimeric mice revealed an important role for α4 integrin in lymphopoiesis (Arroyo et al., 1996, 1999). These mice, which lack both α4β1 and α4β7 integrins, showed an early block in B cell development and had defective T cell precursors that were unable to leave the BM. In addition, no erythrocytes derived from α4-deficient progenitors and only few myeloid cells were found in adult mutants. Since β7 knockout mice have a normal hematopoiesis (Wagner et al., 1996), these data suggested that α4β1 plays a crucial role for the development of hematopoietic progenitors (Wagner and Müller, 1998). We now show that β1 integrins including α4β1 are not essential for lymphopoiesis, erythropoiesis, or myelopoiesis. Our analysis of B cell development in BM and spleen did not reveal any gross abnormality in the differentiation of these cells. One explanation for this finding could be that α4β1 and α4β7 have redundant functions in early hematopoiesis and only the absence of both molecules impairs hematopoiesis. A double knockout of β1 and β7 should allow confirmation of this hypothesis. However, differences in the expression pattern (Voura et al., 1997) and function of α4β1 and α4β7 argue against this hypothesis. For example, α4β1 but not α4β7 can promote activation of T cells binding fibronectin or VCAM-1, indicating that α4β1-specific signaling responses lead to T cell activation (Lehnert et al., 1998). Encephalitogenic T cells express both α4β1 and α4β7. However, only antibodies against α4β1 or VCAM-1, but not against α4β7, diminished the severity of the disease (Engelhardt et al., 1998).

An alternative explanation is that loss of β1 integrin receptors in addition to α4β1 rescues the α4 null phenotype. The decrease in transmigration through the stroma observed with α4 null progenitor cells could for example be due to an increased adhesion to fibronectin via α5β1. Numerous studies of integrin-integrin crosstalks have been described in the literature (Blystone et al., 1999, and references therein).

The migration of T cell precursors from the BM to the thymus was proposed to be at least partially mediated

by α6β1 integrin (Ruiz et al., 1995). Furthermore, adhesion and migration of thymocytes within the thymus was suggested to be dependent on a coordinated engagement of α4β1 and α5β1 (Salomon et al., 1994; Crisa et al., 1996). We found that β1 null T cell precursors are able to migrate to and develop normally within the thymus. However, we observed a temporary reduction in the number of thymocytes 5 weeks after induction of the knockout, which was confirmed by a decreased number of β1 null T cells in the fetal thymi 4 weeks after engraftment under the kidney capsule. Since the relative numbers of thymocyte subpopulations were similar in mutant and control mice, the reduced number of thymocytes could be caused by a less efficient production or emigration of β1-deficient T cell precursors in the BM or migration to the thymus. It is possible, therefore, that α4β1 or α6β1 are important for efficient emigration and migration of T cell precursors, respectively. The transitory nature of the phenotype can be explained by a reduced requirement of the thymus for precursors, but also indicative of a compensatory mechanism "repairing" the defect.

Lymphocyte Migration

Lymphocytes extravasate by weakly binding to and rolling on the endothelium, followed by integrin activation, integrin-mediated firm adhesion, and transmigration through the endothelial cell layer and the underlying basal lamina ([BL]; Moser and Loetscher, 2001). Although β1 integrin is crucial for the adhesion of HSC to the endothelium of hematopoietic organs (Hirsch et al., 1996; Potocnik et al., 2000), it was not known whether it plays a similar role on differentiated lymphocytes. Since antibodies against the BL component laminin reduced lymphocyte emigration to lymph nodes in vivo (Kupiec-Weglinski and De Sousa, 1991) and adhesion to laminins correlated with the ability of T cells to transmigrate these barriers (Sixt et al., 2001), it was suggested that β1 integrin-mediated binding of extravasating cells to BL components like laminin is crucial for this process. We could not confirm a role of β1 integrin in lymphocyte trafficking, suggesting that β1 integrin on lymphocytes is dispensable for firm adhesion to and transmigration through endothelium and BL. Competitive lymphocyte migration assays to spleen, lymph nodes, and Peyer's patches did not reveal significant differences in the migration of β1-deficient and normal cells. Apparently, β1-mediated adhesion is dispensable for lymphocyte adhesion to endothelium of lymphoid organs and for the transmigration through the endothelial cell layer. It is possible that the migration through the BL does not require binding to this structure, but rather local protease activity enabling cell movement without specific contact of cellular receptors and BL molecules (Friedl and Brocker, 2000).

Chemokine-mediated upregulation of integrin affinity plays an important role in lymphocyte trafficking (Moser and Loetscher, 2001). Targeted disruptions of genes for chemokines or chemokine receptors, as for example CCR7 (Förster et al., 1999) and CXCR5 (Förster et al., 1996), cause defects in the homeostatic traffic of lymphocytes but also in the location of lymphocytes within the secondary lymphoid tissues. Conceivably, all these migratory effects of chemokines involve integrin activity. β1 integrins, however, seem not to be essential for the function of these homeostatic cytokines, as documented by the normal migration behavior and organization of the secondary lymphoid organs in mice with β1-deficient lymphocytes. Further studies will be necessary to determine whether specific subsets of B and T cells are affected by the loss of β1 integrin expression.

Immune Response

The normal development of a primary TD immune response is associated with an early production of serum IgM and a later rise in IgG. The isotype switch away from IgM is controlled by cell-cell contact between T helper and B cells, by cytokines secreted by the helper cells and by the number of divisions the B cell undergoes. In fact, IgG-expressing plasmablasts can be detected quite early in extrafollicular foci (Toellner et al., 1998). A number of mice have been described that are unable to switch isotype and produce only IgM, including mice deficient in CD40, OCA-B, and Vav1 (Kawabe et al., 1994; Kim et al., 1996; Gulbranson-Judge et al., 1999). In contrast, no selective defect in the IgM response has been reported to date and, so the β1 integrin-deficient mice exhibit a unique phenotype, secreting very little IgM after immunization with a TD antigen (DNP-OVA), but instead an increased amount of IgG subclasses. The signals that drive the IgM response to protein antigens are not known; indeed, this response has often been viewed as relatively T cell independent, almost a default pathway. The results presented here indicate that this is not the case and that specific cell interactions are required to generate the primary IgM. The enhanced levels of IgG in these mice demonstrate that switch signals are available and that the loss of β1 integrin does not cause a general attenuation of the T cell-dependent immune response. Summarized simply the data suggest that a cell interaction vital for the initiation or maturation of the IgM response is impaired in the β1 integrin-deficient mice. The notion of a simple coactivator role of β1-integrin on T cells, which was proposed based on in vitro experiments (Yamada et al. 1991; Damle and Aruffo, 1991), seems to be ruled out by these results.

A number of models could explain this phenotype. First, signals necessary for the IgM response, normally delivered during T-B cell interaction in the T zones of secondary lymphoid tissues (Liu et al., 1992; Jacob and Kelsoe, 1992), are missing in the β1 integrin null chimeras. This might include interactions with accessory cells as well as direct T-B interactions. Second, the lack of β1 integrin signals could lead to increased delivery of switch signals (by T cells or dendritic cells [DC]). It should be noted that both IgG1 (Th2) and IgG2a (Th1) are increased and so there is no skewing of the response to Th1 or Th2 in these mice, suggesting no obvious change in the cytokine switch signals. Finally, the differentiation or survival of IgM secreting plasma cells is impaired in these mice, either directly due to lack of β1 integrin signaling in B cells or indirectly because of a β1 integrin requirement in the control of cell interactions or production of survival factors from T cell or DC. This last hypothesis cannot explain the increased amount of IgG observed in the mutant mice.

We favor the first interpretation, although it is not mutually exclusive with the second. If correct, this

strongly predicts that the signals necessary for initial B cell activation and IgM secretion are delivered in a quite distinct microenvironment from those that control isotype switching. This conflicts with recent data suggesting that switch signals are delivered very early after, and hence in the same sites as, initial B cell activation (Toellner et al., 1998). Preliminary investigation of the IgM defect by transfer of wild-type B cells and/or T cells into nonirradiated β1 null BM chimeras showed that neither B nor T cells could rescue the impaired IgM response (data not shown). This suggests that β1 integrin is not involved in the direct collaboration of B and T cells but possibly is important for the interaction of B or T cells with an accessory cell. In relation to this, it is interesting to note that dendritic cells in the spleens of β1-deficient chimeras showed a complete absence of β1 integrin expression, in contrast to the incomplete deletion in the lymphocyte compartment (see above). Future adoptive transfer analyses will focus on reconstitution of the dendritic cells in these mice.

When we studied the response of the β1 integrin null BM chimeras to a TI-2 antigen (DNP-Ficoll) we found both the IgM and IgG3 responses were impaired, although the loss of IgM was not as severe as in the TD response. TI-2 antigens are large, repeating epitope polysaccharides that stimulate antibody production by extensive and prolonged crosslinking of B cell receptors. It has been proposed that a second signal is required for optimal activation and differentiation to plasma cells and some have been identified; e.g., interferon-γ (Snapper et al., 1992) and TACI (von Bulow et al., 2001; Yan et al., 2001). Both B1 B cells and marginal zone (MZ) B cells take part in TI-2 responses (Martin et al., 2001), in sites distinct from the T-dependent response. The results shown here could indicate that either a β1 integrin acts as a direct costimulus for B cells in TI-2 responses or that a cell interaction is lacking in the mutant mice due to aberrant adhesion/migration. Interestingly, NK and NK-T cells have been implicated in TI-2 responses (Mond et al., 1995) and both cell types express high amounts of β1 integrin (L. Fahlén and C.B., unpublished data). A deficiency in β1 integrin may prevent delivery of NK- or NK-T cell-derived help to CD1-expressing B cells. It is also possible that the T-dependent and T-independent defects have a common cause. For instance, the expansion of B cell blasts and their differentiation into plasma cells in the extrafollicular foci located in the red pulp are events common to both types of response (Garcia de Vinuesa et al., 1999). This is certainly true for TD IgM and TI-2 IgM and IgG, while TD IgG responses may be propagated elsewhere (e.g., BM). β1 integrin may be important for the formation of these foci. The β1 integrin null chimeras are a unique resource, allowing for the first time a systematic dissection of the lymphocyte-accessory cell interactions that occur during the early T-dependent antibody response, as well as those involved in TI responses.

Experimental Procedures

Generation of Mice with a Deletion of the β1 Integrin Gene in the Hematopoietic System

Mice with a deletion of the β1 integrin gene in the hematopoietic system were generated as described previously and bred in a conventinal animal facility (Nieswandt et al., 2001).

Southern Blotting

Preparation of genomic DNA and Southern blotting were carried out according to standard procedures. Genomic DNA was digested with EcoRI and probed with a fragment of the lacZ gene, which detects only the targeted allele. Scanning was carried out using the AlphaImager program (Alpha Innotech, San Leandro, USA).

Flow Cytometry

Single cell suspension were prepared by gently pushing the dissected organs through 70 μm cell strainers (BD). FACS analysis was performed as described (Potocnik et al., 2000). The following antibodies were used: hamster anti-β1 integrin (Hα2/5), rat anti-β2 integrin (C71/16), rat anti-β3 integrin (2C9.G2), rat anti-β4 integrin (346-11A), rat anti-β7 integrin (M293), hamster anti-α1 integrin (Ha31/8), hamster anti-α2 integrin (Ha1/29), rat anti-α4 integrin (R1-2), rat anti-α5 integrin (5H10-27), rat anti-α6 integrin (GoH3; all Pharmingen), mouse anti-α7 integrin (obtained from K. von der Mark, Erlangen, Germany), rat anti αv-integrin (H9.2B8), rat anti-B220 (RA3-6B2), rat anti-CD19 (1D3), rat anti-CD25 (7D4), rat anti-IgM (R6-60.2), rat anti-IgD (11-26c.2a), rat anti-CD21 (7G6), rat anti-CD23 (B3B4), rat anti-CD4 (H129.19), rat anti-CD8 (53-6.2), rat anti-TCR (H57-597), rat anti-CD44 (IM7), rat anti-Gr-1 (RB6-8C5), rat anti-Mac-1 (M1-70), Ter-119, rat anti-CD62L (MEL-14), rat anti-CD69 (H1.2F3), rat anti-CD3 (17A2), rat anti-Ly-5.1 (A20), rat anti-Ly-5.2 (104), rat anti Ly-9.1 (30C7; all Pharmingen). Antibodies were unconjugated or conjugated with FITC, PE, or biotin and used at 1:200 dilution. Unconjugated antibodies were detected by FITC-conjugated rat anti-hamster IgG (G70-204,G94-56, Pharmingen, 1:200 dilution), FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-mouse IgG (both from Jackson Immunoresearch, 1:200 dilution). Biotinylatyed antibodies were detected by streptavidin PE (Southern Biotechnology, 1:2000 dilution) or streptavidin Cy-5 (Jackson Immunoresearch, 1:500 dilution).

Colony Formation Assay

Blood was collected from the retroorbital bulbus of mutant and control mice. Erythrocytes were lysed with ACK buffer (Coligan et al., 1995). 600000 (PB) or 60000 (BM) leukoytes in 300 μl Iscove's MDM with 2% FCS were mixed with 3 ml MethoCult GF M3434 (StemCell Technologies, Vancouver, Canada), containing Epo, IL-3, IL-6, and SCF, and plated into three 35 mm culture dishes. Colonies were counted after 8–9 days incubation at 37°C, 5% CO_2. preB cell colony assays were performed similarly using 50000 BM leukocytes per dish and MethoCult M3630 (StemCell Technologies) containing IL-7.

T Cell Migration Assay

BM cells from (fl/+ cre) or (fl/– cre) mice (Ly-9.1$^+$) were depleted by MACS (Miltenyi Biotech) for lineage marker positive cells and injected into lethally irradiated C57BL/6J-IghaThy1aGpi1a recipients (10000 cells/mouse). 4 weeks after transfer, β1 integrin deletion was induced as described above. 4 weeks later, 3–4 alymphoid lobes from E15.5 C57BL/6 Ly-5.1 mice (Jenkinson et al., 1982) were transplanted under the kidney capsule of β1-deficient BM chimeras with at least 95% knockout. 4–5 weeks after engraftment the cellular composition of the thymi was analyzed by FACS.

Lymphocyte Homing

Lymphocytes were isolated from spleen of polyIC treated (fl/– cre) or control mice (both Ly-5.2). After lysis of erythrocytes, T and B cells were purified by MACS (Thy-1.2 or B220 beads; Miltenyi Biotech). The purity was about 90% in the enriched and 85% in the depleted fractions. Lymphocyte fractions of control and knockout mice, one of them labeled with the intracellular tracking dye CMTMR (Molecular Probes, USA), were mixed at a ratio of 1:1 and injected into the tail vein of B6SJL (Ly-5.1) mice. After 4 or 16 hr, lymphocytes were isolated from spleen, lymph nodes (inguinal, axial, paraaortic), and Peyer's patches. The ratio of control to mutant cells in these organs was determined by FACS using Ly-5.2 staining to specifically detect the transferred cells. In a second experiment, splenocytes were directly labeled with CMTMR, mixed with unlabeled cells, and injected into recipient mice. FACS analysis was carried out by simultaneous staining with antibodies against Ly-5.2 and either B220 or CD3.

To assess the localization of the transferred cells within the tissue,

either mutant or control lymphocyte fractions were injected. Transferred Ly-5.2$^+$ cells were detected on cryo-sections by immunostaining.

Immunofluorescence

Immunofluorescence was performed as described (Körner et al., 2000). In addition to antibodies described for flow cytometry biotinylated rat anti-mouse CD90.2 (clone 53-2.1, Pharmingen; T24, ATCC), sheep anti-mouse IgD (The Binding Site, Birmingham, UK) and texas red conjugated goat anti-mouse IgM (Southern Biotechnology) were used. The staining was developed with goat anti-rat Alexa488, goat anti-rat Alexa594, streptavidin-Alexa488, streptavidin-Alexa594 (all Molecular Probes), streptavidin texas red (Southern Biotechnology), FITC-conjugated Fab mouse anti-rat IgG (Jackson Immunoresearch), and streptavidin-Cy3 (Jackson, Immunoresearch). Germinal centers were identified by FITC conjugated PNA (Vector laboratories). NP(nitrophenyl)-specific plasma cells were detected on acetone-fixed cryostat sections using NP conjugated to sheep IgG (a gift from Prof. Ian MacLennan, University of Birmingham, UK), which in turn was detected with donkey anti-sheep IgG (The Binding Site, Birmingham, UK) and then streptavidin-FITC (Southern Biotechnology).

Immunization

Mice were immunized via the intraperitoneal route with 100 μg of alum precipitated dinitrophenylated (DNP) ovalbumin (OVA) (Sigma) or with 100 μg of DNP-Ficoll (Biosearch Technologies, USA). ELISA assays were carried out as described (Gray et al., 1994).

In Vitro Stimulation of B Cells

Splenic leukocytes were cultured in 24-well plates at 1.5×10^6 cells/well, 1 ml/well in RPMI 1640, 10% FCS, 50 μM 2-mercaptoethanol, 10 mM Hepes (pH 7.4), 2 mM glutamin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin, in the presence or absence of 10 μg/ml LPS (L9023, Sigma). Cell supernatants were analyzed by ELISA.

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